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13. ABSTRACT (Maximum 200 Words)

Many clinical studies have shown that apoptosis may be related to various pathological parameters of breast cancer, such as tumor size, histologic features, metastasis, and survival. Over 50% of human breast cancer biopsies show amplification or overexpression of *c-myc*, an oncogene that is known to play a crucial role in cell proliferation, apoptosis, and transformation. Female *c-myc* transgenic mice also develop mammary cancer that is characterized by a large number of apoptotic cells, thus serving as a good in vivo model for study on the role of c-Myc in both mammary carcinogenesis and apoptosis. On the other hand, TGFα, a growth factor also frequently overexpressed in human breast cancer, has been shown in MT-*tgfα*/MMTV-*c-myc* double transgenic mice to enhance c-Myc-induced mouse mammary carcinogenesis, probably in part by blocking apoptosis. Our proposal set out to examine the c-Myc mechanisms of mediated apoptosis in our mouse mammary tumor model. In addition, we examined the survival-promototing effect of the TGFα-EGF receptor pathway in this model. We initially provided evidence for TGFα-EGF receptor-mediated cell survival by a calcium/calmodulin mediated pathway regulating Akt. Subsequent studies have focused on the role of a recently discovered kinase, PNCK, and p55γ, a pI3 kinase subunit, to mediate the EGF survival signal.

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Table of Contents

Introduction

Apoptosis is a type of cell death that has been programmed in a cell for its elimination, when it is no longer needed. Many clinical studies have shown that programmed cell death may be related to various biologic parameters of breast cancer, such as tumor size, morphologic features, metastasis, and survival. Over 50% of human breast cancer biopsies show amplification or overexpression of *c-myc*, an oncogene that is known to play a crucial role in cell proliferation, apoptosis, and malignant transformation. Mice carrying a *c-myc* transgene also develop mammary cancer that is characterized by a large number of apoptosis cells. These data suggest that *c-myc* may play an important role in the breast cancer formation and in apoptosis of the cancer cells and that the *c-myc* transgenic mouse may serve as a good *in vivo* model for the study of these aspects of breast cancer. On the other hand, TGFα, a growth factor also frequently overexpressed in human breast cancer, has been shown in *tgfα/c-myc* double transgenic mice to enhance c-Myc-induced mammary cancer development, probably in part by blocking apoptosis. Our initial data showed that in *c-myc* transgenic mammary tumors, several apoptosis-promoting proteins, including cytochrome c (Cyt-c), Apaf-1, and caspase-9, that form a complex, termed the apoptosome, were expressed mainly in the apoptotic cells. However, caspase-3, one of 3 enzymes that executes apoptosis, upon activation by the apoptosome, was not expressed at detectable levels in the apoptotic cells. On the other hand, AIF, which does not need to be activated by caspase to induce apoptosis, was found in the nuclei of apoptotic cells. *We initially hypothesized* that c-Myc-induced apoptosis in *c-myc* transgenic mammary tumors may involve AIF and the apoptosome, but not the executor caspases. Our preliminary data also showed that caspase-8, a mediator of several extracellular signals for apoptosis, was abundantly expressed and present as both activated and inactivated forms. However, it was localized mainly to the major tumor areas that were not apoptotic. *We therefore had also hypothesized* that in *c-myc* transgenic mammary tumors, c-Myc-induced apoptosis may involve activation of caspase-8, but that in most c-Myc-expressing, non-apoptotic cells, this pathway is blocked somewhere downstream of caspase-8. The caspase-8-mediated and the apoptosome-mediated pathways may cross-talk with each other, possibly *via* a link between Cyt-c and caspase-8. Moreover, since apoptotic cells rarely appeared in mammary tumors from *tgfα/c-myc* double transgenic mice, we had also initially hypothesized that, in the double transgenic mammary tumors, TGFα blocks c-Myc-induced apoptosis by inhibition of activation of certain caspase-independent factors, such as AIF, and/or by inhibition of the factors that block the downstream events of caspase-8 and/or of the apoptosome.

In our proposed studies, we were to conduct a dual approach to understanding the mechanisms of c-Myc-induced apoptosis and TGFα - induced survival; first, *in vitro*, using our cell line models. Next, we had proposed to utilize an *in vivo* transgenic tumor model, to further validate results. Specifically, in the first Aim, we were to examine the effect of EGF withdrawal from our c-Myc-expressing mouse mammary tumor model on apoptosis, dependent upon either AIF or executor caspases. We would then follow-up any *in vitro* positive results, showing executor caspase-independent apoptosis, with *in vivo* testing. In the second Aim, if caspase 8 were involved, we would evaluate the possible role of caspase 8 *in vitro*, followed by *in vivo* studies. In the final Aim, we would explore the mechanism of EGF/TGFα promoted survival of c-Myc-expressing, pro-apoptotic cells. However, for Aims 1 and 2, we found that, contrary to our hypothesis, c-Myc-promoted apoptosis depended upon executor caspases but not on caspase 8. In addition, for Aim 3, we made novel observations implicating Ca++/ calmodulin and a recently described Ca++/calmodulin kinase termed pregnancy-upregulated non-ubiquitous CaM kinase (Pnck) in activation of Akt, to mediate EGF/TGFα-promoted survival. We then proposed and had approved by the U.S. Army Medical Research and Materiel Command a revised statement of work to further examine these novel findings, with special reference to Ca++/Calmodulin and to Pnck.

Body

In the proposed grant, we were to address the following Statement of Work:

Task 1: Determine whether c-Myc-induced apoptosis in *c-myc* transgenic mammary tumors involves AIF and the apoptosome (Cyt-c, Apaf-1, and caspase 9), but not the executor caspases – 3, -6, or –7.

Status: Negative results obtained, implicating executor caspases. Task completed.

In Task 1, we began by studying the possible role of executor caspases in c-Myc-induced apoptosis. Although we had not expected involvement of executor caspases, we found that EGF withdrawal from our Myc 83 (c-Myc expressing cell model) induced cleavage of PARP, an executor caspase substrate. This cleavage was blocked by treatment of cells with z-VAD-funk, a broad specificity caspase inhibitor. Based on these results, we concluded that the underlying hypothesis of Task 1 was incorrect, and that there was nothing particularly unusual about c-Myc-induced apoptosis, relative to executor caspase utilization. We therefore deprioritized the *in vivo* part of the task, and moved on to Task 2.

Task 2: Determine whether caspase –8 is involved in c-Myc-induced apoptosis in *c-myc* transgenic mammary tumors and determine how it is related to the apoptosome-mediated pathway.

Status: Negative results obtained, ruling out caspase 8. Task completed.

In Task 2, we employed a specific inhibitor of caspase 8, z-LETD-funk, to test the involvement of caspase 8 in the apoptosis induced by EGF withdrawal of Myc 83 cells. Results were again negative, indicating no involvement of caspase 8. However, when cells were treated with FASL, a known, proapoptotic inducer of caspase 8, apoptosis was inhibited, as expected, by z-LETD-funk. Accordingly, the hypothesis underlying Task 2 was shown to be incorrect, and we again de-prioritized *in vivo* follow-up experiments, moving on to Task 3.

Task 3: Study the mechanisms by which TGFα blocks the c-Myc-induced apoptosis in MT-*tgfα*/MMTV-*c-myc* double transgenic mammary tumors.

Status: Positive results, implicating Akt in the EGF receptor-mediated survival pathway, with the unexpected implication of Ca++/calmodulin signaling in the process. Revised Statement of Work [3a,b below] was approved in 2003 by the DOD, to capitalize on these findings.

Subtask 3a: Determine, using *PNCK* gene transfection into mouse mammary epithelial cells, whether this kinase functions primarily to block proliferation or to promote apoptosis.

Subtask 3b: Determine, using yeast 2-hybrid methodology what are major substrates of Pnck. We will then compare these substrates to our existing c-Myc expression database for design of further work to explore anti-tumor mechanisms of Pnck in mammary cancer.

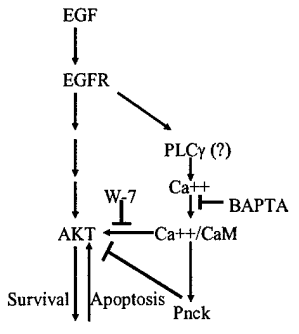
Status: Revised subaims in progress.

In Task 3, we initially set out to study survival mechanisms, engaged by EGF/TGFα treatment of c-Myc-expressing mouse mammary tumor cells. We completed a study demonstrating that EGF receptor activation activates AKT and upregulates $BclX_L$. Pharmacologic inhibition of PI3K/AKT and MEK/Erk signaling pathways partially inhibited induction of apoptosis, in association with downregulation of Akt and Erk1/2 protein levels. We also used a constitutively activated Akt (myr-Akt) to inhibit apoptosis in the same system, confirming a survival-promoting role of Akt. To further address the mechanism of EGF receptor-dependent activation of Akt, we decide to explore the possibility of PI3-K and MEK/Erk – independent

pathways of activation. This objective was pursued by testing of a variety of common signal transduction inhibitors on our EGF-promoted, Myc 83 cell survival model. Surprisingly, we found that EGF-mediated Akt activation (and cell survival) was nearly abolished by calcium chelation and by inhibitors of Ca++/calmodulin. We have now completed and published a full description of this phenomen implicating a novel function of calcim-calmodulin in modulating Akt-dependent cell survival (2).

Calcium ion, an important intracellular signaling molecule, exerts may of its signaling properties by interaction with a calcium binding protein called calmodulin. Calmodulin, coupled with calcium ion is able to interact with and activate a variety of kinases, termed calcium-calmodulin dependent protein kinases (CaM kinases). CaM kinases are extensively studied in the nervous system, where they play a critical role in intra-neuronal signaling, and in muscular tissue, where many of their substrates have been identified including cytokeletal proteins, such as actin and myosin. Their role in mammary epithelium signaling was virtually unknown, until a few years ago. Today, little is know about the role of CaM kinases in the breast. A few CaM kinases, however, are expressed in both normal and transformed mammary epithelial cells.

Pnck, a newly identified calcium/calmodulin kinase, is reported to be specifically expressed in MMTV-c-Myc transgenic mouse mammary tumor cells (3,4). Calcium and its ubiquitous sensor, calmodulin, are important intracellular signaling molecules that participate in a variety of cellular pathways including growth regulation, transcription and even neurotransmitter release. Our recent investigations revealed that calmodulin is overexpressed in epithelial cells derived from MMTV-c-Myc transgenic mouse mammary tumor (5). Previous studies from different laboratories, convincingly demonstrated that calcium is released from intracellular stores in response to growth factors in mammary epithelial/carcinoma cells. These observations, coupled with our observation of upregulation of calmodulin in MMTV-c-Myc cells, prompted us to investigate the role of calcium/calmodulin in cellular survival in MMTV-c-Myc cells. Calmodulin is known to positively regulate neutrophin/BDNF-induced survival in neuronal cells. The initial observations reported that Pnck is upregulated *in vitro*, in over-confluent and serum starved cells, compared to actively growing mammary epithelial cells. They suggest that Pnck expression is inversely related to mammary epithelial cell proliferation or may have a role as a negative regulator of cellular survival/proliferation. *In vivo*, Pnck is upregulated during certain stages of mammary gland development, specifically in late pregnancy and during post-lactational involution when epithelial cells are undergoing terminal differentiation, with decreased proliferation. We reasoned that before investigating the functional aspects of Pnck in mammary epithelial cell survival, we should first investigate the survival mechanism in these cells (Model 1). Accordingly, a series of investigations on survival signaling were carried out. MMTV-c-Myc cells were found to be sensitized to apoptosis in the absence of serum and growth factors. Addition of epidermal growth factor (EGF) rescued these apoptotic cells to survival from apoptosis (5). Further investigation on pro-survival nature of EGF revealed that it activates pro-survival kinase Akt, since constitutively active Akt (myr Akt) when overexpressed, also rescued apoptotic cells in absence of EGF (5). EGF-induced Akt activation was found to be PI-3 kinase (6), calcium and calmodulin dependent since LY294002 (PI-3 kinase inhibitor), BAPTA-AM (calcium chelator) and W-7 (calmodulin antagonist) all inhibited EGF-induced Akt activation. Biologically, apoptosis could be induced by LY294002 (5) and by W-7 in MMTV-c-Myc cells, further implying that PI-3 kinase and calmodulin-mediated Akt activation ais an integral part of the cell survival mechanism. In order to investigate whether calcium/calmodulin-mediated Akt activation occurs in other mammary epithelial cells, other non-tumorigenic human mammary epithelial cells, along with their c-Myc overexpressing counterparts were tested for W-7 sensitivity on EGF-induced Akt activation. In all of these cell lines tested, EGF-induced Akt activation was inhibited by W-7, implying that calmodulin may be a central regulator of survival mechanism in mammary epithelial cells, irrespective of tumorigenicity, species, and c-Myc expression level. Furthermore, Akt activation by other survival ligands, such as insulin, fetal bovine serum (FBS) were



Model 1: Possible role of Pnck in survival signaling

also inhibited by calmodulin antagonist W-7, indicating that other survival factors including EGF, also transduce survival signaling through calmodulin. These data are now all published by us and represent key progress supported by this grant (2).

Since EGF-induced Akt activation is connected to both PI-3 kinase and calmodulin in MMTV-c-Myc cells, and since calmodulin was previously reported to activate PI-3 kinase, we investigated whether these two events were interconnected. Calmodulin-sepharose specifically pulled down the non-ubiquitous PI-3 kinase regulatory subunit ($p55^{PIK}$) in a calcium dependent manner in an *in vitro* assay (Fig.1). Classical, ubiquitous p85 regulatory subunit was not detected in calmodulin-sepharose precipitates. The binding of calmodulin to $p55^{PIK}$ could be inhibited by EGTA. EGF-indiced Akt activation was inhibited by the PLC-γ inhibitor, U-73122. This observation suggests that an EGF-induced and calcium/calmodulin-dependent PI-3 kinase activation, leading to Akt activation, may be present in these cells. Neither the calmodulin antagonist W-7 nor U-73122 could inhibit binding of ubiquitous PI-3 kinase regulatory subunit, p85, to phosphotyrosine. *In vitro,* a PI-3 kinase assay further revealed that W-7 has no effect on EGF-induced PI-3 kinase activity associated with anti-phosphotyrosine and anti-p85 immunoprecipitates. Currently, we are examining how calmodulin-associated PI-3 kinase activity is affected by W-7. Our observations suggest that both classical phosphotyrosine / p85 dependent PI-3 kinase activity and a PLC-γ /calcium/calmodulin-$p55^{PIK}$ -mediated PI-3 kinase activity are operating, converging on AKT activation and survival of these c-Myc overexpressing breast cancer cells (Model 1). We also examined the possible mechanism(s) downstream of calmodulin and upstream of Akt in MMTV-c-Myc cells. Since calmodulin kinases are the probable candidates, we employed a pharmacological inhibitor approach. STO-609 (CaM kinase kinase inhibitor), KN-62 (CaM kinase II inhibitor) and Rottlerin (CaM kinase III inhibitor) could not inhibit EGF-induced Akt activation. These results suggest that calmodulin-mediated Akt activation is not mediated through any of these three known calmodulin kinases. Pnck, a recently described Ca/CaM kinase, has strong homology with CaM kinase I at the catalytic domain, and is the subject of our continued study.

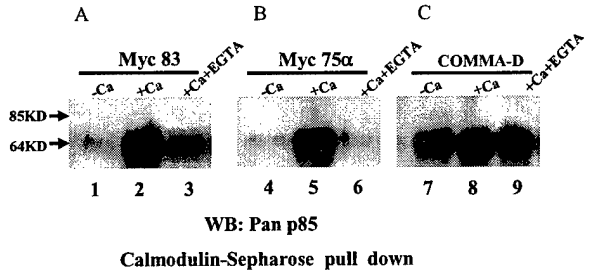


Figure 1. Calmodulin binds to $p55^{PIK}$/p55γ. Serum starved Myc 83 (A) Myc 75α (B) and Comma D (C) cells were lysed in EDTA-free lysis buffer, and 1 mg/ml total proteins were incubated with 30μl calmodulin-sepharose suspension for 2 h at 4 °C with or without 100 μM $CaCl_2$ (lanes 1-2, lanes 4-5, lanes 7-8). A third lysate from each cell line was incubated with 100 μM $CaCl_2$ in the presence of 1mM EGTA (lanes 3, 6 and 9). Calmodulin-sepharose beads were probed with a pan p85 antibody (p85α/β/$p55^{PIK}$/p55γ). Control-sepharose suspension did not precipitate $p55^{PIK}$/p55γ in calcium dependent manner (data not shown). $P55^{PIK}$/p55γ migrates with an eletrophoretic mobility of 64KDa on SDS-PAGE.

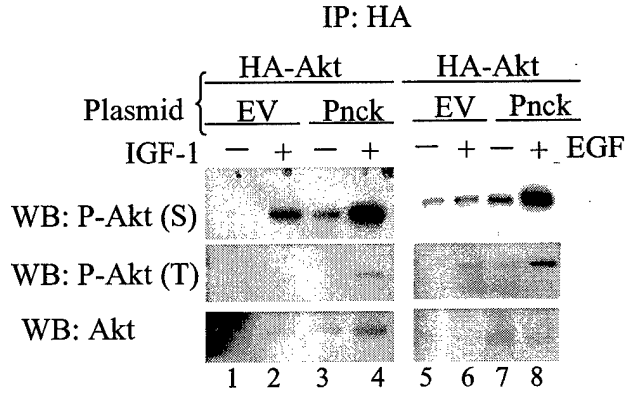


Figure 2. Ectopic Pnck expression augments IGF-I/EGF-induced Akt activation in MCF-7 cells. MCF-7 cells were transiently co-transfected with empty vector (EV) and HA-Akt (lanes1-2; lanes5-6) or Pnck construct and HA-Akt (lanes 3-4; lanes 7-8). Cells were serum-starved and stimulated with or without IGF-I (lanes 1-4) or EGF (lanes 5-8), lysed and HA-Akt was immunoprecipitated by anti-HA mAb (12CA5). Immunoprecipitates were probed for activation specific (S-473P/T-308P) Akt antibodies.

Pregnancy Up-regulated Non-ubiquitous Cam kinase (Pnck), an unique calmodulin kinase and the subject of the final year of this grant, is upregulated in the mouse mammary gland, specifically during late pregnancy and post-lactational involution, where it is expressed in a specific spatio-temporal pattern during mammary gland development. The chromosomal localization (Xq28) and mouse cDNA sequence of Pnck, have been published. Pnck is also expressed in an oncogene-associated manner; specifically, it is upregulated in cell lines derived from transgenic MMTV-*c-Myc* mammary tumors, but not those from transgenic MMTV-*ras* or MMTV-*neu* mouse. However, whether it is acutely upregulated by c-Myc is not known. Based on comparative mRNA expression studies, which demonstrated that Pnck mRNA levels were high in confluent and serum starved mammary epithelial cells, compared to actively growing and serum stimulated cells, it was predicted that Pnck might be a negative regulator of the cell cycle or of cell survival. In striking contrast to this prediction, however, Pnck mRNA was shown to be upregulated in primary human breast cancers, but not in adjacent benign breast tissue. RNA protection assays also revealed Pnck mRNA in a variety of human breast cancer cell lines. However, no biochemical/functional data yet exist in the scientific literature to support the anti-proliferative /anti-survival role of Pnck.

To examine a potential role of Pnck in calmodulin-mediated Akt activation process, we have cloned human *PNCK* in a mammalian expression vector, and we have developed a polyclonal antibody against this protein. This antibody specifically detects both endogenous and exogenously expressed human Pnck in breast cancer cell lines (data not shown) and in Cos-7 cells, respectively. Preliminary data suggest that when *PNCK* cDNA is transiently co-expressed with an *HA-Akt* plasmid in human MCF-7 cells, both EGF- and IGF-I-dependent Akt activation is strongly potentiated (Fig. 2). These data enabled us to hypothesize that Pnck, probably functions as a positive mediator of Akt activation. These data, as well as a 3-5-fold up-regulation of Pnck in human breast cancer, prompted us to believe that Pnck might be a good target for future breast cancer therapy. Akt activation provides crucial survival signals in breast epithelium and its deregulated function is a strong indicator of breast oncogenesis. Although Akt activation is predominantly an indicator of cell survival, in some cases Akt activation might lead to apoptosis and differentiation. Currently, we do not know whether potentiated Akt activation in MCF-7 or other human breast cancer cells augments cell survival or counteract survival. Accordingly, we will conduct research to investigate biological outcome and mechanism of Pnck mediated Akt activation. The final year of this grant will fully address this question, in accordance with Tasks 3a, b of our revised and approved statement of work.

Our study advances DOD Breast Cancer Research program objectives because the better understanding of mechanisms of growth factor-mediated cell survival will contribute to better diagnosis, prognosis and/or therapy of the disease. For example blockade of a calcium-calmodulin kinase target (such as Pnck) could provide an effective new therapeutic target of the disease.

Key Research Accomplishments

1. Determination that c-Myc-promoted apoptosis in c-Myc-initiated transgenic mouse mammary tumor cells depends upon executor caspases, but not caspase 8.

2. Determination that EGF-mediated survival of c-Myc-initiated transgenic mouse mammary tumor cells is partially mediated by PI3-K/Akt and MEK/Erk pathways.

3. Discovery that EGF-mediated survival of c-Myc-initiated transgenic mouse mammary tumor cells is largely dependent upon Ca++/calmodulin signaling to regulate Akt membrane localization, potentially *via* Pnck, a recently discovered, c-Myc-regulated, Ca++/calmodolin-dependent protein kinase.

4. Establishment of a database (and publication of) c-Myc-induced genes in MMTV-c-Myc transgenic mouse mammary tumors, to serve as a resource for experiments to determine the possible survival-regulatory role of Pnck in this mammary tumor type.

5. Discovery of EGF/Ca++/calmodulin-mediated regulation of survival through induction of p55γ, a regulatory subunit of phosphatidyl-3-kinase.

6. Discovery that Pnck expression potentiates EGF-mediated activation of Akt in MCF-7 human breast cancer cells.

Reportable Outcomes

Papers Published:

1. Desai KV, Xiao N, Wang W, Gang L, Greene J, Powell JI, Dickson RB, Furth P, Hunter K, Kucherlapati R, Simon R, Liu ET, and Green JE, Initiating oncogenic event determines gene expression patterns of human breast cancer models, Proc Nat'l Acad Sci (USA), 99:6967-6972, 2002.

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3. Ramljak D and Dickson RB, Cell signaling in the breast, in Dennis and Bradshaw (eds), Handbook of Cell Signaling, Academic Press, 345:565-571, 2003.

4. Deb TB, Coticchia CM, and Dickson RB, Calmodulin-mediated activation of Akt regulates survival of c-Myc over-expressing mouse mammary carcinoma cells. J. Biol. Chem., 279:38903-38911, 2004.

Abstracts Published:

1. Ramljak D, Coticchia CM, and Dickson RB, Epidermal growth factor receptor signaling inhibits c-Myc-induced apoptosis through activation of Akt, Erk, and upregulation of BclXL in mouse mammary carcinoma cells, AACR Symposium on Apoptosis, Keystone Co, 2001.

2. Desai KV, Xiao N, Wang W, Gangi L, Greene J, Powell JI, Dickson RB, Furth P, Hunter K, Kurherlapati R, Simon R, Liu ET, and Green JE, Probing oncogenic pathways in transgenic mouse models of mammary cancer by cDNA microarray analysis, Proceedings of the AACR, San Francisco, CA, 2002.

3. Ramljak D, Coticchia CM, Nishanian TG, and Dickson RB, AKT inhibits c-Myc-mediated apoptosis in mammary epithelial cells: a mechanistic investigation, DOD ERA of Hope Meeting, Orlando, FL, 2002.

4. Deb TB, and Dickson RB, Calmodulin as a positive modulator of EGF survival signaling in MMTV-c-MYC mouse mammary epithelial cells, DOD ERA of Hope Meeting, Orlando, FL, 2002.

Conclusions

1. c-Myc-promoted apoptosis in c-Myc-initiated transgenic mouse mammary tumor cells depends upon executor caspases, but not caspase 8.

2. EGF-mediated survival of c-Myc-initiated transgenic mouse mammary tumor cells in partially mediated by PI3-K/Akt and MEK/Erk pathways.

3. EGF-mediated survival of c-Myc-initiated, transgenic mouse mammary tumor cells is largely dependent upon Ca++/calmodulin signaling to regulate Akt membrane localization.

4. EGF-mediated survival of c-Myc initiated, mouse mammary tumor cells may proceed through induction of $p55^{PIK}$/p55γ, a regulatory subunit of phosphatidyl-3-kinase and through activation of Pnck, a c-Myc-regulated, Ca++/calmodulin-dependent protein kinase.

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2. Deb TB, Coticchia CM, and Dickson RB, (2004) Calmodulin-mediated activation of Akt regulates survival of c-Myc over-expressing mouse mammary carcinoma cells. J. Biol. Chem., 279:38903-38911.

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Appendix

1. Deb TB, Coticchia CM, and Dickson RB, Calmodulin-mediated activation of Akt regulates survival of c-Myc over-expressing mouse mammary carcinoma cells. J. Biol. Chem., 279:38903-38911, 2004.

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Calmodulin-mediated Activation of Akt Regulates Survival of c-Myc-overexpressing Mouse Mammary Carcinoma Cells*

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c-Myc-overexpressing mammary epithelial cells are proapoptotic; their survival is strongly promoted by epidermal growth factor (EGF). We now demonstrate that EGF-induced Akt activation and survival in transgenic mouse mammary tumor virus-c-Myc mouse mammary carcinoma cells are both calcium/calmodulin-dependent. Akt activation is abolished by the phospholipase C-γ inhibitor U-73122, by the intracellular calcium chelator BAPTA-AM, and by the specific calmodulin antagonist W-7. These results implicate calcium/calmodulin in the activation of Akt in these cells. In addition, Akt activation by serum and insulin is also inhibited by W-7. EGF-induced and calcium/calmodulin-mediated Akt activation occurs in both tumorigenic and non-tumorigenic mouse and human mammary epithelial cells, independent of their overexpression of c-Myc. These results imply that calcium/calmodulin may be a common regulator of Akt activation, irrespective of upstream receptor activator, mammalian species, and transformation status in mammary epithelial cells. However, only c-Myc-overexpressing mouse mammary carcinoma cells (but not normal mouse mammary epithelial cells) undergo apoptosis in the presence of the calmodulin antagonist W-7, indicating the vital selective role of calmodulin for survival of these cells. Calcium/calmodulin-regulated Akt activation is mediated directly by neither calmodulin kinases nor phosphatidylinositol 3-kinase (PI-3 kinase). Pharmacological inhibitors of calmodulin kinase kinase and calmodulin kinases II and III do not inhibit EGF-induced Akt activation, and calmodulin antagonist W-7 does not inhibit phosphotyrosine-associated PI-3 kinase activation. Akt is, however, co-immunoprecipitated with calmodulin in an EGF-dependent manner, which is inhibited by calmodulin antagonist W-7. We conclude that calmodulin may serve a vital regulatory function to direct the localization of Akt to the plasma membrane for its activation by PI-3 kinase.

One of the fundamental etiologic processes in tumorigenesis is the ability of cancer cells to evade programmed cell death, or apoptosis (1). Potent cell survival signaling, in parallel with uncontrolled cell proliferation and other processes, ultimately leads to the development of a malignant tumor. In breast cancer, this pathologic outcome is strongly influenced by growth factors and/or hormones, which interact with their cognate receptors on mammary epithelial cells. Receptor-ligand interactions at the cell surface are propagated as cascades of signals through the cytoplasm, culminating in specific gene expression programs in the nucleus to define specific biological outcome(s). The serine/threonine kinase Akt is considered a central player controlling cellular survival (2), apoptosis (3), and oncogenesis (4, 5). Akt is activated by growth factors and other stimuli, through both phosphatidylinositol 3-kinase (PI-3 kinase)[1]-dependent and independent mechanisms (6–9). PI-3 kinase, a ubiquitous lipid kinase and upstream effector of Akt (10), has also been implicated in a variety of cellular functions, including survival and antiapoptosis (11, 12), growth and proliferation (13, 14), differentiation (15, 16), cytoskeletal rearrangement (17), translocation of glucose transporter GLUT4 (18, 19) and membrane ruffling (20). Upon growth factor stimulation, PI-3 kinase generates 3′-phosphorylated phosphoinositides, such as phosphatidylinositol 3,4-biphosphates and phosphatidyl inositol 3,4,5-triphosphates, at the plasma membrane. These phosphoinositides serve as binding anchors for the Pleckstrin homology domain of Akt and thus encourage translocation of Akt to the plasma membrane (10, 21–23). At the plasma membrane, Akt is phosphorylated at Ser-473 and Thr-308 and fully activated (24). In addition to a PI-3 kinase-dependent mechanism of Akt activation, a PI-3 kinase-independent mechanism(s) of Akt activation has also been reported. The prime candidate mediating this mechanism is calmodulin kinase kinase, which directly phosphorylates Akt in a calcium-dependent manner (6). Calmodulin, the allosteric regulator of calmodulin kinases, also regulates PI-3 kinase-dependent Akt activation, independent of calmodulin kinase kinase, and is known to control neuronal cell survival (25, 26) and GLUT4 translocation in 3T3-L1 adipocytes (27). Calmodulin binds to the p85α regulatory subunit of PI-3 kinase (28), but this binding does not result in the generation of phosphatidyl inositol 3,4,5-phosphates, which are required for membrane targeting of Akt and for its subsequent activation (10). A calcium/calmodulin-dependent PI-3 kinase (hVPS34) cascade, responsible for phagosome maturation, has recently been reported (29). A consensus sequence in the p110 catalytic subunit of PI-3 kinase has been predicted to be the binding site of calmodulin, but no biochemical data currently exist to support this idea (30). Be-

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[1] The abbreviations used are: PI-3 kinase, phosphatidylinositol 3-kinase; MMTV, mouse mammary tumor virus; FBS, fetal bovine serum; EGF, epidermal growth factor; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl ester; MAP, mitogen-activated protein; pAb, polyclonal antibody; PARP, poly(ADP-ribose) polymerase; PI, phosphatidylinositol; PKC, protein kinase C; EF-2, elongation factor 2; PLC, phospholipase C; EGFR, epidermal growth factor receptor.

cause mammary epithelial cells can release abundant calcium from intracellular stores in response to growth factors and survival ligands (31, 32), we hypothesized that calcium plays a major role in survival of these cells. Our laboratory has already reported that EGF-induced survival of c-Myc-overexpressing mammary carcinoma cells is mediated by activation of PI-3 kinase/Akt kinase (33). In search of a specific survival mechanism downstream of EGFR, which may be a therapeutic target in breast carcinoma, and to uncover any existing relationship between PI-3 kinase and calcium mediated activation of Akt, we undertook further investigation of calcium- and PKC-dependent survival mechanism(s). Our new studies resulted in the identification of a calcium/calmodulin-dependent Akt activation and survival mechanism in these cells. In particular, EGF-induced Akt activation is mediated by calmodulin, the universal calcium sensor, resulting in cell survival. We have shown that calmodulin does not exert its effect directly at the PI-3 kinase level. We have further shown that an EGF-dependent complex forms between calmodulin and Akt. This mechanism probably transports Akt to the plasma membrane for its activation by a PI-3 kinase-dependent mechanism. Perturbation of this targeting mechanism by calmodulin antagonism leads to apoptotic cell death in tumorigenic mammary carcinoma cells. This novel mechanism may have broader implications in the regulation of breast cancer, GLUT4 translocation, and neuronal survival.

EXPERIMENTAL PROCEDURES

Cell Lines—Mammary tumor-bearing MMTV-c-Myc transgenic and MMTV-c-Myc/MT-TGFα bitransgenic mice have been described previously (34–36). Mammary tumor-derived carcinoma cells from these mice were cultured in improved modified Eagle's minimum essential medium containing 2.5% fetal bovine serum (FBS), 10 ng/ml EGF, and 5 μg/ml insulin. The MMTV-c-Myc transgenic tumor cell lines Myc83, Myc9, and Myc7 were examined, and representative experiments were performed in Myc83 cell lines. Likewise, Mycα75 was chosen as a representative cell line of MMTV-c-Myc/MT-TGFα bitransgenic tumors. The Comma D cell line was previously derived from normal mammary epithelium (37–38); cells were cultured in improved modified Eagle's minimum essential medium containing 5% FBS. Isolation and maintenance of non-tumorigenic immortal human mammary epithelial cell lines 184A1N4 and 184A1N4-Myc were described previously (39, 40). A description of retroviral transfection, selection, and development of immortalized MCF 10A-LXSN and MCF 10A-c-Myc stable human mammary epithelial cell lines was published previously (41).

Antibodies and Reagents—Pharmacological inhibitors staurosporine, BAPTA-AM, GF109203X, W-12, W-7, KN-62, KN-92, AG1478, and Rottlerin were purchased from Calbiochem. STO-609 was from Tocris Cookson Inc. Phospho-Akt (Ser-473), Phospho-Akt (Thr-308), phospho-p44/42 MAP kinase (Thr-202/Tyr-204) and phospho-EF2 (Thr-56) and Akt pAb were obtained from Cell Signaling Technology, Inc. Akt 1(C-20) goat polyclonal Ab, PARP pAb (H-250), and CaM I (FL-149) pAb were purchased from Santa Cruz Biotechnology, Inc. U-73122, *N*-benzyloxycarbonyl-VAD-fluoromethyl ketone, and immobilized bovine calmodulin on Sepharose were purchased from BIOMOL Research Laboratories. α-Tubulin, Ab-2 monoclonal antibody was from NeoMarkers, Inc. Pan-extracellular signal-regulated kinase monoclonal antibody was obtained from BD Biosciences. Monoclonal calmodulin antibody, p85α pAb, anti-phosphotyrosine monoclonal antibody (clone 4G10), and biotin-conjugated anti-phosphotyrosine (clone 4G10) were purchased from Upstate Biotechnology. Hoechst stain was from Sigma. Phosphatidylinositol was purchased from Avanti Polar Lipids.

Treatment with Pharmacological Inhibitors and Preparation of Whole Cell Lysates—Semiconfluent cell monolayers were serum-starved overnight and then incubated with inhibitors for indicated periods of time. Cells were stimulated with 10 nM EGF for 3 min or for the indicated time periods (as shown in the text and figure legends) (in time course experiments) at 37 °C, and lysed in lysis buffer (10 mM Tris-base, pH 7.4, 1% Triton-X-100, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml each of pepstatin, leupeptin, and aprotinin). Lysates were vortexed and centrifuged at 15,000 $\times$ *g* for 15 min at 4 °C. Protein concentrations of lysates were measured using BCA protein assay kit (Pierce) and Ultramark Microplate Imaging System (Bio-Rad).

Immunoprecipitation and Western Blotting—Immunoprecipitations and Western blotting were performed as described previously (42). In brief, 1 μg of antibody was added to 500 μg of clarified whole cell lysates and incubated for 1 h at 4 °C. 5 μl of protein A-agarose beads were added, and lysates were further incubated for 1 h at 4 °C. Beads were precipitated by centrifugation at 15,000 $\times$ *g* for 2 min and washed 3 times in lysis buffer. Bound proteins were released by boiling in SDS-PAGE sample buffer for 3 min. Proteins were resolved on SDS-PAGE and transferred to polyvinylidene difluoride (Immobilon-P; Millipore) membranes. Membranes were incubated in primary antibody for 2 h, followed by biotinylated secondary antibody for 1 h, and detected by Vectastain ABC Elite kit (Vector Laboratories) and enhanced chemiluminescence (PerkinElmer Life and Analytical Sciences).

Hoechst Staining and PARP Cleavage Apoptosis Assays—Semiconfluent growing cells were transferred to complete medium containing either 30 μM W-12 or W-7. After 36 h, all floating and adherent cells were collected. Samples were centrifuged for 8 min at 1000 $\times$ *g* at 4 °C. Supernatants were discarded, and cell pellets were suspended in a 1$\times$ phosphate-buffered saline solution containing 0.3% formaldehyde and 2% Nonidet P-40 and stained with 10 μg/ml of Hoechst 33258 dye (Sigma) for apoptotic analysis. For each replicate, at least 500 cells were counted and evaluated for the presence of condensed nuclei and overall apoptotic appearance. For PARP cleavage assays, a third plate was treated with W-7, in the presence of 50 μM *N*-benzyloxycarbonyl-VAD-fluoromethyl ketone, a broad-spectrum caspase inhibitor. After 16 h, adherent cells were trypsinized and lysed, and equal amounts of total proteins were resolved in SDS-PAGE and immunoblotted by anti-PARP antibody.

PI-3 Kinase Assay—PI-3 kinase assays were performed, using a modified protocol from Kapeller *et al.* (44). In brief, anti-phosphotyrosine and anti-p85α immunoprecipitates were washed twice in lysis buffer (phosphate-buffered saline, pH 7.5, 1% Nonidet P-40, and 100 μM vanadate), twice in a second buffer (10 mM Tris-Cl, pH 7.5, 100 mM NaCl, 100 μM LiCl, and 100 μM vanadate), and finally twice in a third buffer (10 mM Tris-Cl, pH 7.5, 100 mM NaCl, and 100 μM vanadate). Lipid kinase assays were performed on washed beads at 37 °C for 10 min in a reaction mixture containing 20 mM HEPES, pH 7, 40 μM ATP, 10 mM $MgCl_2$, 100 μM vanadate, 0.2 μg/μl sonicated phosphatidylinositol (PI), and 20 μCi of [γ^{32}-P]ATP (6000 μCi/mmol). At the end of the reaction, 80 μl of HCl (1 M) were added, followed by 160 μl of methanol/chloroform (1:1), and the organic layer was extracted and spotted on oxalate/EDTA-preactivated thin layer chromatography plates. Thin layer chromatography plates were resolved using a running buffer containing 60:40:11.3:2 chloroform/methanol/H_2O/ammonium hydroxide.

RESULTS

EGF-induced Akt Activation Is Inhibited by Staurosporine but Not by GF109203X—Our previous observations implicated EGF-induced PI-3 kinase/Akt activation as a survival mechanism of MMTV-c-Myc mouse mammary carcinoma cells (henceforth called Myc83 cells) (33). We next wanted to investigate the possible upstream signaling mechanisms regulating this Akt kinase activation and cell survival. In a previous study with small cell lung cancer cells, c-Myc sensitized these cells to apoptosis during nutrient depletion (45). Under these conditions, PKC-β2 overexpression improved cell survival by protecting against c-Myc-induced apoptosis. PKCδ is also known to promote survival of small cell lung cancer cells (46). By analogy, we hypothesized that EGF-induced PKC activation, upstream of PI-3 kinase, might be responsible for Akt activation. To begin to test this idea, serum-starved Myc83 cells were incubated with staurosporine, a broad spectrum PKC inhibitor, as well as GF109203X, a specific inhibitor of PKC (all isoforms) (47–49), for 30 min, followed by stimulation with 10 nM EGF for 3 min. Cells were lysed, and equal amounts of total protein-containing lysates were probed by activation specific anti-phospho Akt (Ser-473) antibody. Although 1 μM staurosporine completely inhibited EGF-induced Akt activation, 5 μM GF109203X had no effect (Fig. 1*A*, *WB: P-Akt S-473*). 5 μM GF109203X also failed to inhibit EGF-induced Akt activation in serum-starved, bitransgenic MMTV-c-Myc/MT-TGFα mouse

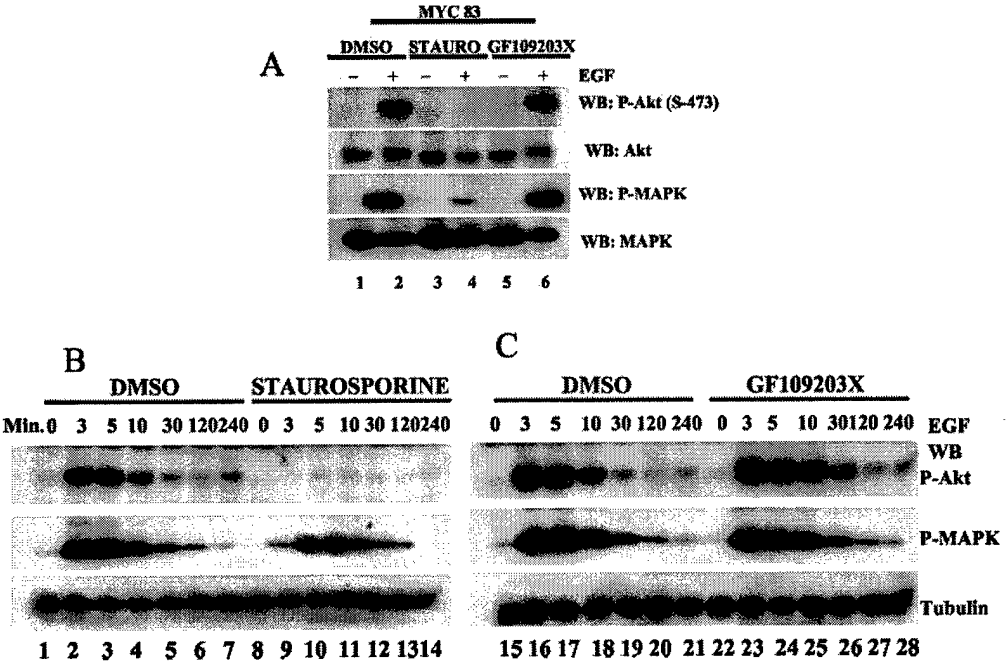


FIG. 1. **Effect of staurosporine and GF109203X on EGF-induced Akt activation.** *A*, staurosporine, but not GF109203X, inhibits EGF-induced Akt activation in Myc83 cells. Serum-starved cells were preincubated with Me_2SO (*lanes 1* and *2*), 1 μM staurosporine (*lanes 3* and *4*), and 5 μM GF 109203X (*lanes 5* and *6*) for 30 min and treated with or without 10 nM EGF for 3 min. Equal amounts of total protein containing cell lysates from each sample were resolved on SDS-PAGE and transferred to polyvinylidene difluoride membrane. Membrane was probed by anti-phospho Akt (Ser-473) antibody [*WB: P-Akt (S-473)*] or anti-phospho-MAP kinase antibody (*WB: P-MAPK*). Each blot was reprobed by anti-Akt (*WB: Akt*) and anti-MAP kinase antibody (*WB: MAPK*), respectively. *B* and *C*, time course experiment reveals sustained Akt inhibition but transient MAP kinase inhibition by staurosporine but not by GF109203X. Serum-starved cells were incubated with Me_2SO (*DMSO*) and 1 μM staurosporine (*B*) or 5 μM GF109303X (*C*) and stimulated with 10 nM EGF for indicated time period. Lysates were probed for Akt (*WB: P-Akt*) and MAP kinase activation (*WB: P-MAPK*) as described in *A*. Each blot was reprobed by anti-tubulin antibody (*WB: Tubulin*) for consistent loading.

mammary carcinoma cells (henceforth called Mycα75 cells) and non-malignant mouse Comma D cells (37–38) (data not shown). Staurosporine also caused significant inhibition of MAP kinase. However, GF109203X had no effect on MAP kinase activity (Fig. 1A, *WB: P-MAPK*). 5 μM GF109203X inhibited PMA-induced MAP kinase activation in all of these cell lines, indicating that it is active in these cells at this concentration (data not shown). To examine whether GF109203X inhibits Akt activation at later time points, the effect of both staurosporine and GF109203X on Akt and MAP kinase activation were examined in a time course experiment. Staurosporine inhibited Akt activation in a sustained manner, whereas GF109203X did not inhibit Akt activity at any time point tested (Fig. 1, *B* and *C*, *WB: P-Akt*). On the other hand, MAP kinase activity was significantly inhibited by staurosporine at 3 min, but substantial activity was regained at later time points (Fig. 1*B*, *WB: P-MAPK*). Similar to results in the short term experiment, neither EGF-induced Akt activation nor MAP kinase activity was affected by GF109203X in the time course experiment (Fig. 1*C*, *WB: P-Akt* and *WB: P-MAPK*). Taken together, these data show that EGF-induced Akt activation is mediated by a staurosporine inhibitable-factor but not by PKC.

Calcium Chelator BAPTA-AM and Calmodulin Antagonist W-7 Inhibit EGF-induced Akt Activation—Staurosporine has been reported to inhibit a variety of kinases, including calcium/calmodulin kinase II (50) and PKC (51). Considering that mammary epithelial cells release abundant calcium from intracellular stores in response to EGF (31, 32), we hypothesized that a calcium-regulated signaling mechanism downstream of EGFR might be a potential effector of Akt activation. To test this idea, serum-starved cells were preincubated for 90 min with 10 μM BAPTA-AM, an intracellular calcium chelator (52–54), and stimulated with EGF. BAPTA-AM completely inhibited Akt activity and partially inhibited EGF-induced MAP kinase activity (Fig. 2*A*, *WB: P-Akt S-473* and *WB: P-MAPK*), whereas incubation of the cells with 2 mM EGTA (external calcium chelator) affected activation of neither Akt nor MAP kinase (Fig. 2*A*, *WB: P-Akt S-473* and *WB: P-MAPK*). Identical results were obtained in both Myc α75 and Comma D cells (data not shown). These results suggest that EGF-induced release of calcium from intracellular stores is required for Akt activation in Myc83, Mycα75, and Comma D cells.

To further examine whether calcium mediates its effect on Akt activation through the universal calcium sensor calmodulin, we used a selective calmodulin antagonist, W-7 (27, 55). Calmodulin antagonists have been used previously for inhibiting nerve growth factor- and brain-derived neurotrophic factor-induced Akt activation, resulting in neuronal cell survival (25, 26). As shown in Fig. 2*B*, 30-min pretreatment of 30 μM W-7, but not its inactive analogue W-12 (56), significantly inhibited EGF-stimulated Akt activation in Myc83 cells (*WB: P-Akt S-473*). Similar to W-7-treated Myc83 cells, Mycα75 and Comma D cells also showed significant inhibition of their EGF-induced Akt activity (Fig. 3, *B* and *C*, *WB: P-Akt S-473*). The specificity of W-7 was confirmed by its ability to block EGF-induced dephosphorylation of elongation factor 2 (EF-2) (Fig. 3, *A–C*, *WB: P-EF-2*). EF-2 remains highly phosphorylated (Thr-56) in quiescent cells, thus inhibiting peptide chain elongation (57) and protein synthesis. Growth factors, such as insulin, cause dephosphorylation of EF-2 *via* calcium/calmodulin-dependent activation of calmodulin kinase III (formerly known as EF-2 kinase), resulting in peptide chain elongation. The calmodulin antagonist W-7 and the EF-2 kinase inhibitor Rottlerin both effectively inhibit this dephosphorylation. Calmodulin has been implicated in survival of neuronal cells (25, 26) and chicken lymphoma B cells (58), but we are unaware of any report indicating a role for calmodulin in mediating Akt activation linked to mammary epithelial cell survival. Although

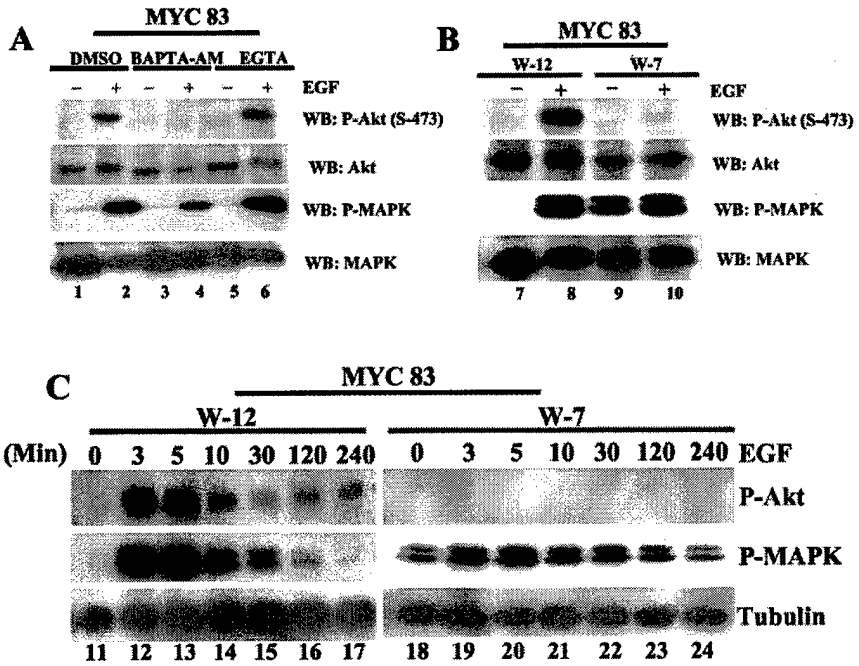


FIG. 2. **Effect of intracellular calcium chelator, BAPTA-AM, and calmodulin antagonist W-7 on Akt activation in Myc83 cells.** *A*, intracellular calcium chelator BAPTA-AM inhibits EGF-induced Akt activation. Myc83 cells were serum-starved overnight and incubated with Me_2SO (*DMSO*; *lanes 1* and *2*), 10 μM BAPTA-AM for 90 min (*lanes 3* and *4*), and 2 mM EGTA (*lanes 5* and *6*) for 90 min followed by 3 min of EGF stimulation. Lysates were probed for activated Akt [*WB: P-Akt (S-473)*] and activated MAP kinase (*WB: P-MAPK*). Blots were reprobed for total Akt (*WB: Akt*) and total MAP kinase (*WB: MAPK*), respectively. *B*, calmodulin antagonist W-7 inhibits Akt activation but up-regulates MAP kinase activation. Serum-starved cells were incubated with 30 μM W-7 (*lanes 9* and *10*) or inactive analogue W-12 (*lanes 7* and *8*) for 30 min. Cells were stimulated with 10 nM EGF for 3 min and processed for Akt and MAP kinase activation as described in Fig. 1*A*. *C*, sustained Akt inhibition and MAP kinase activation by calmodulin antagonist. Serum-starved Myc83 cells were preincubated with 30 μM W-7 (*lanes 18–24*) or inactive analogue W-12 (*lanes 11–17*) for 30 min, and stimulated with 10 nM EGF for indicated time period. Akt and MAP kinase activation were examined as described in Fig. 1*B*. Blots were reprobed by anti-tubulin antibody (*WB: Tubulin*) for consistent gel loading.

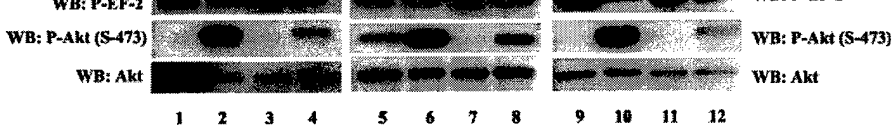


FIG. 3. **Inhibition of Akt activation by calmodulin antagonist occurs in both tumorigenic carcinoma and non-tumorigenic mouse mammary epithelial cells.** Tumorigenic cell lines Myc83 (*lanes 1–4*), Mycα 75 (*lanes 5–8*), and non-tumorigenic cell lines Comma D (*lanes 9–12*) were pre-incubated with either W-7 or W-12 for 30 min, stimulated with 10 nM EGF for 3 min, and processed for Akt activity exactly as described in Fig. 2*B*. An identical blot from each cell line was probed by anti-P-EF-2 (Thr-56) antibody (*WB: P-EF-2*).

calmodulin was shown to function upstream of Akt kinase leading to neuronal cell survival, the intermediate signaling mechanism is not completely understood.

We next examined the ability of calcium/calmodulin to activate Akt in a series of MMTV-c-Myc and MMTV-c-Myc/MT-TGFα transgenic mouse mammary tumor-derived cell lines, in addition to Myc83 and Mycα75, respectively. In all cases, W-7 but not W-12 (inactive analogue) inhibited activation of Akt, thus excluding any effect of clonal variation in this mechanism (data not shown). However, W-7 incubation did not inhibit EGF-induced MAP kinase activity. It is interesting that the basal level of activated MAP kinase, as detected by anti-phospho-MAP kinase immunoblotting, increased in W-7-treated Myc83 cells (Fig. 2*B*, *WB: P-MAPK*). This is in agreement with previous observations of down-regulation of Ras/Raf/ERK pathway by calmodulin and of activation of MAP kinase activity by calmodulin antagonists, observed in NIH 3T3 fibroblasts (43, 59)

To examine whether the effect of W-7 on Akt or MAP kinase activation is sustained, we preincubated Myc83 cells with 30 μM W-7 and then stimulated them with EGF for different time periods. Anti-phospho-Akt (S-473) immunoblotting of the lysates revealed sustained inhibition of Akt activity by W-7, compared with inactive analogue, W-12 (Fig. 2*C*, *WB: P-Akt*). In contrast, W-7 incubation resulted in a sustained MAP kinase activation (Fig. 2*C*, *WB: P-MAPK*). Sustained MAP kinase activation is linked to both proliferation and differentiation, depending upon the cell line. In PC-12 cells, nerve growth factor-mediated, sustained MAP kinase activation results in differentiation (60). In contrast, sustained calmodulin inhibition, in serum-starved fibroblasts cells, induces extracellular signal-regulated kinase 2 phosphorylation and $p21^{cip1}$ expression, leading to inhibition of cellular proliferation (59). Taken together, our data suggest that EGF-induced activation of Akt is mediated by calcium/calmodulin-dependent mechanism(s) and that calmodulin has opposing effects on sustained Akt and

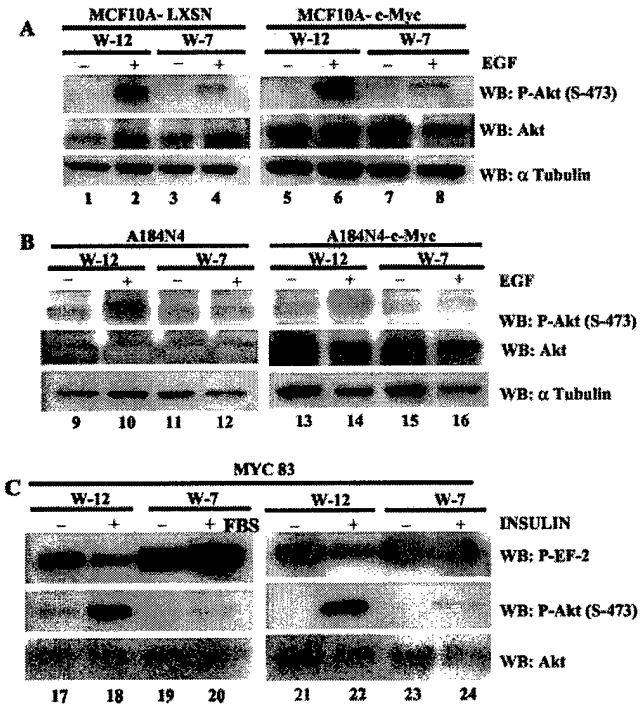


FIG. 4. **Calmodulin antagonism inhibits EGF-induced Akt activation in both c-Myc-overexpressing and normal human mammary epithelial cell lines.** Calmodulin antagonism also inhibits FBS and insulin-induced Akt activation. Human MCF-10A-LXSN (*lanes 1–4*) and MCF-10A-c-Myc (*lanes 5–8*) (*A*), human A184N4 (*lanes 9–12*) and A184N4-c-Myc (*lanes 13–16*) cells (*B*), and mouse Myc83 cells (*C*) were serum-starved and incubated with 30 μM W-7 or W-12 for 30 min and stimulated either with or without 10 nM EGF for 3 min (*A* and *B*), 10% FBS for 5 min (*lanes 17–20*), 100 nM insulin for 5 min (*lanes 21–24*) (*C*). Lysed cells were examined for Akt (*WB: P-Akt S-473*) or EF-2 phosphorylation (*WB: P-EF2*) and total Akt (*WB: Akt*) as described previously.

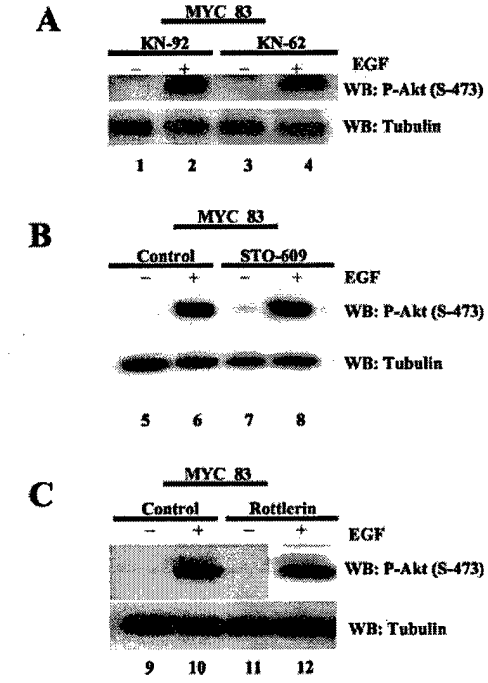


FIG. 5. **Calmodulin kinases do not transduce EGF signal(s) to Akt.** Myc83 cells were serum-starved overnight and incubated with 10 μM KN-62 (*lanes 3* and *4*) or inactive analogue KN-92 (*lanes 1* and *2*) for 2 h (*A*), 100 ng/ml STO-609 (*lanes 7* and *8*) or vehicle (*lanes 5* and *6*) for 6 h (*B*), and 10 μM Rottlerin (*lanes 11* and *12*) or vehicle (*lanes 9* and *10*) for 2 h (*C*). Cells were stimulated with 10 nM EGF for 3 min, and lysates were probed for Akt activity (*WB: P-Akt S-473*) as described before. Each of the blots was reprobed with anti-tubulin antibody (*WB: Tubulin*).

MAP kinase activation in Myc83 cells.

Calmodulin Is a Common Central Regulator of Akt Activation, Irrespective of Ligands, Species, Tumorigenicity, and c-Myc Expression Status—To investigate whether calmodulin-mediated activation of Akt occurs in other mammary epithelial cell systems, we tested the c-Myc-overexpressing, non-tumorigenic human mammary epithelial cell lines 184A1N4-Myc (39, 40) and MCF10A-c-Myc (41), along with their control counterparts 184A1N4 and MCF-10A-LXSN. In all the cell lines tested, W-7 inhibited EGF-stimulated Akt activation (Fig. 4, *A* and *B*, *WB: P-Akt S-473*). W-7 (30 μM) also specifically inhibited insulin- or FBS-induced Akt activation in Myc83 cells (Fig. 4*C*, *WB: P-Akt S-473*). These observations suggest that calcium/calmodulin is a common regulator of Akt activation, irrespective of c-Myc expression status, species, tumorigenicity, and survival ligands in a variety of mammary epithelial cell models.

Calmodulin-mediated Cell Survival Does Not Depend on Calmodulin Kinase Kinase and Calmodulin Kinase II or III—To determine whether calmodulin mediates Akt activation *via* calmodulin kinase(s), we tested specific inhibitors of calmodulin kinases. Serum-starved Myc83 cells were incubated with 100 ng/ml STO-609 for 6 h, 10 μM KN-62 for 2 h, or 10 μM Rottlerin for 2 h, followed by stimulation with 10 nM EGF for 3 min. STO-609 is a potent inhibitor of calmodulin kinase kinase (61, 62), which is an upstream activator of calmodulin kinase I and calmodulin kinase IV (6, 63). KN-62 (inactive analogue KN-92) inhibits calmodulin kinase II (64, 65), and Rottlerin (66) is an inhibitor of calmodulin kinase III (also known as EF-2 kinase). None of these compounds inhibited EGF-induced activation of Akt in Myc83 cells (Fig. 5, *A–C*, *WB: P-Akt S-473*), Mycα75 (data not shown), and Comma D cells (data not shown). Although calmodulin kinase III acts downstream of Akt kinase, and PDK-1-null cells have no calmodulin kinase III activity (67), we used Rottlerin to rule out the possibility of any feedback activation of Akt by calmodulin kinase III. These data suggest that neither calmodulin kinase kinase, calmodulin kinase II, nor calmodulin kinase III transduces EGF-induced, EGFR-originated, and calmodulin-mediated signals to Akt.

Calmodulin Antagonist Does Not Inhibit EGF-induced PI-3 Kinase Activation—Our laboratory previously demonstrated that EGF-dependent survival signaling in Myc83 cells is PI-3 kinase-Akt dependent, because preincubation of cells with PI-3 kinase inhibitor LY294002, inhibited Akt activation, leading to apoptosis (33). Overexpression of constitutively active myr-Akt protected Myc83 cells from LY294002-induced apoptosis (33). Joyal *et al.* (28) have demonstrated that calmodulin binds to the p85α subunit of PI-3 kinase in a calcium-dependent manner. However, this interaction did not produce any phosphatidyl inositol 3,4,5-triphosphate and thus cannot recruit Pleckstrin homology domain-containing proteins, such as PDK1 or Akt, to the plasma membrane (10, 68). A calcium/calmodulin-mediated mechanism of PI-3 kinase (hVPS34) activation was reported recently that describes the mechanism that *Mycobacterium tuberculosis* employs to block phagosome maturation and to evade bactericidal agents (29). To investigate whether calcium/calmodulin-mediated Akt activation is linked to classic EGF-induced PI-3 kinase-Akt activation, mediated by phosphotyrosine-p85 subunits, we employed the specific EGFR tyrosine kinase inhibitor AG1478 (69), the calmodulin antagonist W-7, and the phospholipase C-γ inhibitor U-73122 in Myc83 cells. We then examined whether any of these pharmacological inhibitors affected the ability of p85α regulatory subunit to be co-immunoprecipitated with an anti-phosphotyrosine antibody.

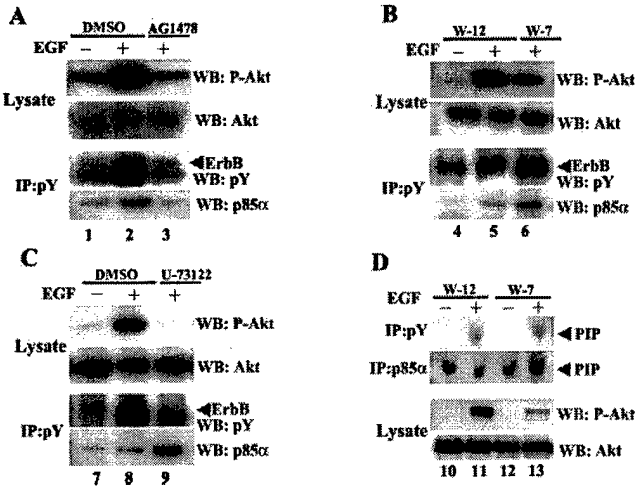


FIG. 6. **Calmodulin antagonist W-7 has no effect on EGF-induced phosphotyrosine-associated PI-3 kinase activities** *A*, EGFR kinase inhibitor AG1478 inhibits both EGF-induced Akt activation and association of p85α with phosphotyrosine. Serum-starved Myc83 cells were pre-incubated with either Me_2SO (*DMSO*) or 1 μM of the specific EGFR tyrosine kinase inhibitor AG1478 for 30 min and then stimulated by EGF (*lanes 2* and *3*), as described previously. Lysates were immunoprecipitated by anti-pY antibody (clone 4G10), and immunoprecipitates were immunoblotted by anti-pY antibodies (clone 4G10) (*WB: pY*) and p85α (*WB: p85α*). Corresponding lysates were probed for activated Akt (*WB: P-Akt*) and total Akt (*WB: Akt*). An *arrow* on the pY immunoblot showed the position of ErbB. *B* and *C*, the calmodulin antagonist W-7 and the PLC-γ inhibitor U-73122 inhibit EGF-induced Akt activation but not the association of p85α with phosphotyrosine. Myc83 cells were serum-starved and pre-incubated with 30 μM W-12 or W-7 (*lanes 4–6 B*) or 10 μM U-73122 (*lanes 7–9 C*) for 30 min and stimulated by EGF. Lysates were immunoprecipitated by anti-pY antibodies as described in *A*. Immunoprecipitates were immunoblotted for pY and p85α (*WB: pY, WB: p85α, B* and *C*). Representative lysates from each experiment were probed for the status of Akt activation and Akt (*WB: P-Akt, WB: Akt, B* and *C*) *D*, the calmodulin antagonist W-7 does not inhibit EGF-induced phosphotyrosine- and p85α-associated PI-3 kinase activities *in vitro*. Immunoprecipitations were performed on Myc83 lysates treated with either W-12 (*lanes 10* and *11*) or W-7 (*lanes 12* and *13*) and stimulated with (*lanes 11* and *13*) or without EGF (*lanes 10* and *12*). *In vitro* PI-3 kinase assays were performed on immunoprecipitates, as described under "Experimental Procedures." Corresponding lyastes were probed for their Akt activation status (*WB: P-Akt*).

Serum-starved Myc83 cells were treated with 1 μM AG1478, 1 μM U-73122, or 30 μM W-7 for 30 min and then stimulated by EGF. Phosphotyrosine-containing proteins from the lysates were captured with an anti-phosphotyrosine antibody, followed by immunoblotting with anti-p85α pAb. As shown in Fig. 6*A*, p85α was co-immunoprecipitated, in an EGF-dependent manner, by anti-phosphotyrosine antibody, but not when cells were pre-incubated with AG1478 (*WB: p85α*). Corresponding lysates from this experiment revealed that EGF-dependent Akt activation was strongly inhibited by AG1478 (Fig. 6*A*, *WB: P-Akt*). Myc83 cells express EGFR and ErbB2 (henceforth called ErbB) and do not express significant ErbB3 and ErbB4 as determined by Western blotting and cellular signaling (data not shown). Reprobing of the p-Tyr immunoprecipitates revealed that AG1478 significantly inhibited EGF-induced ErbB tyrosine phosphorylation (Fig. 6*A*, *WB: pY*). This demonstrates that classic phosphotyrosine-mediated, p85-dependent PI-3 kinase activation is operating in Myc83 cells. Because EGF-induced and calcium/calmodulin-dependent Akt activation require ErbB phosphotyrosine (such as EGFR Tyr-1173) to activate PLC-γ (required for calcium release), it was not possible to separate calcium/calmodulin-dependent Akt activation from phosphotyrosine-dependent, PI-3 kinase-mediated Akt activation by AG1478 treatment. In contrast, the PLC-γ inhibitor U-73122 and the calmodulin antagonist W-7 could not inhibit EGF-dependent pull down of p85α with anti-phosphotyrosine antibody (Fig. 6*B*, *IP: pY/WB: p85α* and Fig. 6*C*, *IP: pY/WB: p85α*), although corresponding lysates revealed strong inhibition in EGF-dependent Akt activity by each of these inhibitors (Fig. 6, *B* and *C*, *WB: P-Akt*). In fact, EGF-dependent p85α binding to phosphotyrosine was increased in cells treated with W-7 (Fig. 6*B*, *IP: pY/WB: p85α*) and U-73122 (Fig. 6*C*, *IP: pY/WB: p85α*).

Calmodulin is known to bind directly to the epidermal growth factor receptor (EGFR) (70, 71), and both calmodulin and calmodulin kinase II were reported to inhibit EGFR kinase activity (70, 72). The calmodulin-binding domain at the juxtamembrane region of EGFR has also been mapped recently (73, 74). In agreement with these observations, W-7 potentiated EGF-induced ErbB tyrosine phosphorylation, probably as a result of up-regulation of EGFR tyrosine kinase activity (Fig. 6*B*, *IP: pY/WB: pY*). Because EGF-dependent p85α association with ErbB is affected by neither W-7 nor U-73122, one could conclude that calmodulin, although associated with EGFR, has no positive effect on association of p85 to ErbB. This indirectly confirms that plasma membrane targeting of functional PI-3 kinase, composed of p85-p110 heterodimer, is not affected by U-73122 or W-7.

To confirm whether calmodulin exerts any effect at the PI-3 kinase level, *in vitro* PI-3 kinase assays were conducted on both anti-p85α and anti-phosphotyrosine immunoprecipitates. EGF-induced PI-3 kinase activities, associated with anti-p85α regulatory subunit and anti-phosphotyrosine immunoprecipitates, could not be inhibited by the calmodulin antagonist W-7 (Fig. 6*D*). We immunoblotted anti-calmodulin immunoprecipitates and calmodulin-Sepharose precipitates by a panel of p85 and pan-p85 antibodies; in every case, no EGF or calcium-dependent association of p85 regulatory subunit with calmodulin was observed (data not shown). We also performed an *in vitro* PI-3 kinase assay on anti-calmodulin immunoprecipitates and calmodulin-Sepharose precipitates, in an EGF- and calcium-dependent manner, respectively, and could detect no PI-3 kinase activity in either case (data not shown). Taken together, these data confirm that calmodulin does not affect targeting of either functional PI-3 kinase or EGF-induced phosphotyrosine-associated PI-3 kinase activities in Myc83 cells.

Calmodulin Forms a Complex with Akt in an EGF-dependent Manner—To investigate whether calmodulin forms complexes with Akt in an EGF-dependent manner, calmodulin was immunoprecipitated from serum-starved and EGF-stimulated Myc83 cells, and immunoprecipitates were probed for bound Akt. As shown in Fig. 7, Akt was co-immunoprecitated with calmodulin in EGF-stimulated Myc83 cells, and this binding was abolished by pretreatment with W-7 (*WB: Akt*). Lysates from the same experiment revealed a parallel inhibition of Akt kinase activities (Fig. 7, *WB: P-Akt S-473*, *WB: P-Akt T-308*). These data suggest that calmodulin forms an EGF-dependent complex by either direct or indirect binding with Akt.

Calmodulin Antagonist W-7 Induces Apoptosis in c-Myc-overexpressing but Not in Normal Mammary Epithelial Cells—To investigate whether calcium/calmodulin-mediated Akt activation contributes to mammary epithelial cell survival, Myc83, Mycα75, and Comma D cells were subjected to calmodulin antagonism by W-7, and cellular apoptosis was studied by PARP cleavage and Hoechst staining. In Myc83 and Mycα75 cells, W-7 treatment induced significant PARP cleavage after 16 h that could be rescued by *N*-benzyloxycarbonyl-VAD-fluoromethyl ketone, a broad spectrum caspase inhibitor. This result confirmed that an early apoptotic program was initiated in Myc83 and Myc α75 cells in response to W-7 (Fig. 8*A*). These

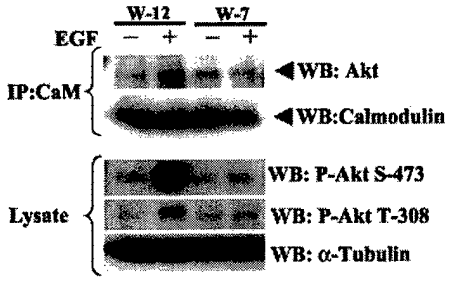
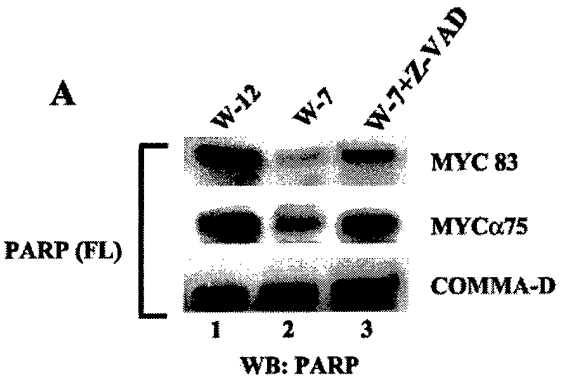


FIG. 7. **Akt is co-immunoprecipitated with calmodulin in an EGF-dependent manner.** EGF-induced co-immunoprecipitation of Akt with calmodulin. Serum-starved, semiconfluent Myc83 cells were pre-incubated with either 30 μM W-12 (*lanes 1* and *2*) or W-7 (*lanes 3* and *4*) for 30 min and induced with or without 10 nM EGF for 3 min. Lysates were immunoprecipitated with an anti-calmodulin monoclonal antibody and then immunoprecipitates were immunoblotted for Akt (*WB: Akt*). The blot was reprobed with an anti-calmodulin pAb to demonstrate consistent immunoprecipitation (*WB: Calmodulin*). Corresponding lysates from this experiment were immunoblotted by anti-Phospho Akt (Ser-473) (*WB: P-Akt S-473*), anti-phospho Akt (Thr-308) (*WB: P-Akt* Thr-308), and by an anti-tubulin antibody (*WB: α-Tubulin*).



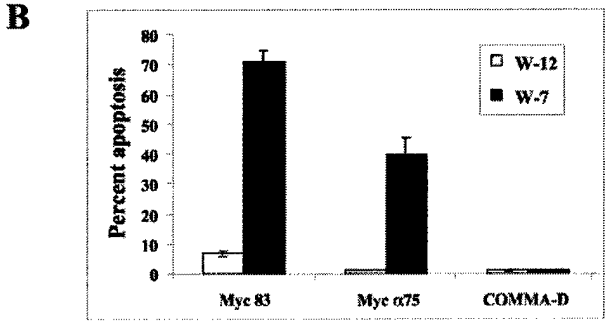


FIG. 8. **Calmodulin antagonist induces apoptosis in Myc83, Mycα75 but not in Comma D cells.** *A*, semiconfluent growing cells were incubated with 30 μM W-12 (*lane 1*) or W-7 (*lane 2*) or 30 μM W-7, in the presence of 50 μM *N*-benzyloxycarbonyl-VAD-fluoromethyl ketone (*Z-VAD*) (*lane 3*) for 16 h. Cells were trypsinized, lysed, and equal amount of total proteins were resolved in SDS-PAGE and immunoblotted by anti-PARP antobody (*WB: PARP*). *B*, semiconfluent growing cells were incubated with 30 μM W-12 or W-7 for 36 h and all floating and adherent cells were stained with 10 μg/ml of Hoechst 33258 dye (Sigma) for apoptotic analysis. For each replicate, at least 500 cells were counted and evaluated for the presence of condensed nuclei and overall apoptotic appearance. Each treatment was conducted in triplicate, and experiments were repeated three times. A representative experiment is presented.

carcinoma cells also showed a very high frequency of apoptosis, measured by Hoechst staining after 36 h of W-7 treatment (Fig. 8*B*). In contrast, Comma D cells did not undergo apoptosis by W-7 in either of these assays (Fig. 8, *A* and *B*). Comma D cells previously showed a marked inhibition of EGF-induced Akt activation by W-7 (Fig. 3*C*). However, this compound had no effect on apoptosis. The simplest explanation for these opposite results is that additional event(s), independent of calmodulin-dependent Akt activation, are required to support survival mechanism of Comma D cells. A recent study demonstrated that immortalization of Comma D cells is independent of the EGF-PLC-PI-3 kinase-Akt signaling cascade (75). Additional future experiments are required to determine the possible contribution of calmodulin to Comma D cell survival.

DISCUSSION

In this communication, we have presented evidence for the existence of a unique mechanism of EGF-induced and calcium/calmodulin-mediated survival in mouse mammary carcinoma cells. EGF-induced activation of Akt was shown to be a prime survival pathway of MMTV-c-Myc transgenic mammary tumor-derived epithelial cells (33). We have shown that EGF-induced and PLC-γ-mediated release of calcium from intracellular stores results in a calcium/calmodulin-dependent activation of Akt and survival of these cells. Calcium/calmodulin-regulated Akt activation in mammary epithelial cells is mediated neither by calmodulin kinases (6) nor directly by a PI-3 kinase-dependent mechanism(s), as described previously for neuronal cells (25). Calmodulin binds to Akt in an EGF-dependent manner, potentially targeting functional Akt to the plasma membrane for its subsequent activation by a PI-3 kinase-dependent mechanism. Calmodulin-mediated Akt activation, therefore, is indirectly linked to a phosphotyrosine-dependent, PI-3 kinase activation mechanism; perturbation of either mechanism by LY294002 (33) or calmodulin antagonist, W-7, induces apoptosis (in this study) in c-Myc-overexpressing mammary carcinoma cells. We also showed that calmodulin regulation of Akt kinase is common in a variety of mammary epithelial cells, irrespective of survival ligands (EGF, insulin, or FBS), c-Myc expression status, species (human or mouse), and tumorigenicity. Calmodulin antagonism specifically resulted in apoptosis of tumorigenic c-Myc-overexpressing mammary carcinoma cells but did not affect normal mammary gland-derived epithelial cells (*i.e.* Comma D), implying that calmodulin-mediated Akt activation is an integral part of the survival mechanism in certain tumorigenic cells.

Calmodulin is a universal calcium sensor and performs a myriad of biological functions including cell growth (76), cell cycle progression, proliferation (77, 78), trafficking (79), synaptic plasticity (80), and glucose transporter GLUT4 targeting to the plasma membrane (27, 81). Cellular incorporation of antisense calmodulin RNA and microinjection of calmodulin antibody leads to cell cycle arrest and inhibition of DNA synthesis (82). Recent publications have highlighted calmodulin's role in modulating cell survival, upstream of Akt kinase, both by PI-3 kinase-dependent or -independent mechanisms. In particular, calmodulin and calmodulin kinase kinase mediate membrane depolarization and, subsequently, cell survival in motor neurons and neuroblastoma cells by a PI-3 kinase-independent mechanism(s) (6, 83). Genetic studies also revealed that calcium/calmodulin, through calmodulin kinase, promotes *Saccharomyces cerevisiae* survival from pheromone-induced growth arrest (84). On the other hand, brain-derived neurotrophic factor- and neurotrophin-induced and calmodulin-mediated cell survival is considered to be mediated by PI-3 kinase-dependent Akt activation (25, 26). Although calmodulin was predicted to control generation of PI-3 kinase products in neuronal cells (25), the exact mechanism has not been addressed. Likewise, translocation of the GLUT4 glucose transporter to the plasma membrane in 3T3L1 adipocytes (27, 81) is regulated by

calcium (85), calmodulin, and Akt, although calmodulin's direct role could not be ascertained (27). In neuronal cells, a calmodulin antagonist inhibited Akt activation, and constitutively active Akt (gag-Akt) expressing neuronal cells escaped apoptosis induced by a calmodulin antagonist (25). In an analogous situation in 3T3-L1 adipocytes, a calmodulin antagonist inhibited insulin-induced Akt activation and GLUT4 translocation to the plasma membrane (27). An enhanced green fluorescent protein-Pleckstrin homology fusion protein also failed to translocate to the plasma membrane in the presence of a calmodulin antagonist. However, in both neuronal cells and 3T3-L1 cells, phosphotyrosine-associated *in vitro* PI-3 kinase was not inhibited by a calmodulin antagonist (25, 27). It was suggested that calcium/calmodulin is probably required for proper *in vivo* targeting of PI-3 kinase to its substrate and calmodulin antagonist inhibits this process. As a result, PI-3 kinase products, such as phosphatidyl inositol 3,4,5-triphosphates, are not produced, and Akt is not activated.

Our investigations are very similar to both of these observations, and we also observe EGF-induced Akt inactivation and apoptosis of Myc83 and Mycα75 cells in the presence of W-7. Similar to neuronal and 3T3-L1 cells, phosphotyrosine-and p85α-associated PI-3 kinase activities were not inhibited by a calmodulin antagonist *in vitro*. In contrast, in our experiments, calmodulin antagonism could not inhibit ligand-induced association between tyrosine phosphorylated ErbB and p85α, implying that targeting of PI-3 kinase to the plasma membrane is not affected. Because membrane targeting of p85 (α/β) alone is not always sufficient for full PI-3 kinase/Akt activity (86), it is possible that the effect of calmodulin on Akt activation is at or distal to PI-3 kinase *in vivo*. Our *in vitro* lipid kinase data demonstrate that the calmodulin antagonist W-7 has no effect at the PI-3 kinase level. However, we observe a calmodulin-Akt association *in vitro* in an EGF-dependent manner that can be disrupted by W-7. This suggests that calmodulin probably performs a trafficking function for Akt by increasing Akt availability to PI-3 kinase products at the plasma membrane. Specific inhibition of either PI-3 kinase activation by LY294002 (33) or of Akt trafficking by a calmodulin antagonist (this study), thus inhibits Akt activation in these cells. A recent study demonstrated that glial cell line-derived neurotrophic factor-induced neuronal survival is mediated by calcium/calmodulin's association with PI-3 kinase, resulting in Akt activation (87). It was shown that calcium-dependent binding of calmodulin to the p85 regulatory subunit induces PI-3 kinase activation, resulting in Akt activation. Previous observations indicate that calmodulin-p85 interaction does not produce phosphatidyl inositol 3,4,5-triphosphate (28), a prime ligand for binding to Akt Pleckstrin homology domain for subsequent Akt activation. In view of this, it is not clear how calmodulin-p85 interaction and its associated PI-3 kinase activity resulted in Akt activation. Calmodulin is up-regulated in Myc83 cells (88), and constitutively activated Akt-expressing neuronal cells escape calmodulin antagonist-induced apoptosis (25). It is established that Akt needs to be plasma membrane-targeted for its activation. However, it is still not clear how Akt is transported to the plasma membrane from the cytoplasm. Because calmodulin forms a complex with Akt, it is more likely that calmodulin regulates Akt targeting and its consequent activation downstream of PI-3 kinase (Fig. 9).

EGF and related ligands, such as TGFα, β-cellulin, and amphiregulin, as well as the ErbB family receptors, have enormous influence on normal mammary development. In addition, dysregulation of either ligands or their receptors is frequently observed in breast cancer (89–92). Calmodulin is up-regulated in a variety of transgenic mouse mammary tumor models, including MMTV-c-Myc (88). Our data demonstrate that calmodulin is a major contributory factor in Akt activation and cellular survival in c-Myc-overexpressing mouse mammary carcinoma cells. Furthermore, EGF-induced Akt activation was also strongly inhibited by W-7 in the human breast cancer cell line MCF-7 (data not shown), indicating that this mechanism is prevalent in human cancer cell lines that do not overexpress c-Myc. Previously, inhibition of calmodulin with W-7 and W-13 in the estrogen receptor-negative breast cancer cell line, MDA-MB-231, prevented colony formation in soft agar, suggesting that inhibition of calmodulin inhibits the transformation processes in certain human breast cancer cell lines independent of estrogen receptor status (93). Although we have shown that calmodulin forms a complex with Akt in an EGF-dependent manner, it is not known whether this interaction is direct or mediated by any auxiliary protein. The IQ motif is known to bind calmodulin in both a calcium-dependent and -independent manner (94). Based on hydropathy, hydrophobic residue, residue charge and mass, α-helical class, and position of particular residue, numerous calmodulin-binding proteins have been described previously (calcium.uhnres.utoronto.ca/ctdb/ctdb/home.html). In that context, Akt might potentially be a calmodulin-binding protein. In contrast, because EGF-induced Akt activation is also inhibited by staurosporine, it is probable that a staurosporine-sensitive, auxiliary protein kinase, other than PKC, mediates calmodulin's association to Akt. Our future investigations are directed toward investigating the interaction of Akt with calmodulin and associated survival signaling. These findings could have biologic relevance for defining the phenotype(s) of c-Myc-overexpressing breast cancer.

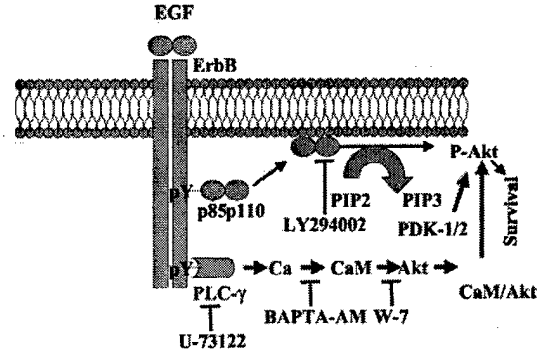


FIG. 9. **A model of calcium/calmodulin-dependent Akt activation and survival in Myc83 cells.** A probable model of calcium/calmodulin-induced Akt activation, and cell survival is presented. EGF-induced activation of ErbB tyrosine kinase produces phosphotyrosines, which can serve as binding ligands for Src homology 2-domain-containing proteins, such as the p85 regulatory subunit of PI-3 kinase and PLC-γ. The p85-p110 heterodimer is thus targeted to the plasma membrane, where PI-3 kinase is activated. Activated PLC-γ increases cytosolic calcium from intracellular stores. Calcium-bound activated calmodulin (*CaM*) associates with Akt and transports Akt to the plasma membrane, where Akt binds to PI-3 kinase products, such as phosphatidyl inositol-3,4,5-triphosphate (*PIP3*), and is subsequently activated by phosphorylation. Inhibition of PI-3 kinase (by LY294002), PLC-γ (by U-73122), chelation of intracellular calcium (by BAPTA-AM), and inactivation of calmodulin (by W-7) all result in Akt inhibition and apoptosis in Myc83 cells.

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